

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/12, 15/63, A23L 3/3463 A01N 37/18	A1	(11) International Publication Number: WO 91/13982 (43) International Publication Date: 19 September 1991 (19.09.91)
(21) International Application Number: PCT/US91/01335 (22) International Filing Date: 28 February 1991 (28.02.91) (30) Priority data: 489,186 8 March 1990 (08.03.90) US (71) Applicant: FERRODYNAMICS, INC. [US/US]; 17400 El Camino Real, Suite 4, Houston, TX 77054 (US). (72) Inventor: KRUZEL, Marian, L. ; 12959 Newbrook, Houston, TX 77072 (US). (74) Agents: PLAYER, William, E. et al.; Wegner, Cantor, Muller & Player, 1233 20th Street, N.W., Suite 300, P.O. Box 18218, Washington, DC 20036-8218 (US).		(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BG, BR, CA, CH, CH (European patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GB, GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SU. Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: GENETICALLY ENGINEERED HUMAN LACTOFERRIN (57) Abstract Disclosed is human lactoferrin-expressed from recombinant DNA, its method of production and purification and its use.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	BS	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

GENETICALLY ENGINEERED HUMAN LACTOFERRIN

The present invention relates to human lactoferrin. In particular, it relates to human lactoferrin, its production, and its use.

5 Lactoferrin plays an important role in iron transport and utilization in humans. A glycoprotein containing about 6% sugar and having a total molecular weight of about 80,000 daltons, human lactoferrin is capable of binding two ferric ions with high affinity. The binding constant of iron by
10 lactoferrin is about 10^{20} M^{-1} . Although lactoferrin binds iron tightly, the binding is reversible so that the metal is available upon demand to cells with a need for this essential element.

Human milk is high in lactoferrin content. The high degree
15 of iron absorption from human milk is manifested in a low incidence of iron deficiency anemia among breast fed infants compared to infants fed with cow's milk. Accordingly, lactoferrin is a key protein for healthy development of infants. However, production of lactoferrin from human milk
20 poses problems. First, the severely limited amount of human milk, the major source of human lactoferrin, that is available restricts lactoferrin production. Secondly, production of lactoferrin from human milk presents a tremendous risk factor of infectious contamination. That is,
25 it could carry with it a potentially lethal contaminant, such as the AIDS virus, or another undesirable agent.

Accordingly, the present invention provides human lactoferrin expressed from recombinant DNA. The lactoferrin of the present invention is free of naturally occurring
30 contaminants, e.g., proteins and viruses, that would be detrimental to the recipient. The present invention also provides a genetically altered organism capable of producing human lactoferrin. In a further aspect, the present invention provides a method of producing human lactoferrin
35 comprising the steps of (a) isolating DNA encoding human

lactoferrin from a cDNA library derived from human breast tissue;

(b) inserting the isolated DNA into the DNA of a host organism; (c) culturing the host organism to express human
5 lactoferrin; and (d) recovering the expressed lactoferrin from the culture media. In still another aspect of the present invention there is provided a process for inhibiting microbial growth in a mammal comprising topically applying to the subject a therapeutically effective amount of
10 lactoferrin having less than 25% metal loading, a process for inhibiting a trace-element deficiency in a mammal comprising orally administering a therapeutically effective amount of lactoferrin having at least 35% trace-element loading. The present invention also provides a nutritional
15 supplement comprising the trace-element loaded human lactoferrin having at least 35% metal loading and a nutritionally acceptable carrier or diluent. The present invention further provides a topical antiseptic comprising an effective amount of lactoferrin having less than 25% metal
20 loading and a pharmaceutically acceptable carrier or diluent. Still another aspect of the present invention provides a process for inhibiting food spoilage comprising adding to the food an effective amount of lactoferrin having less than 25% metal loading.

25 The present invention also contemplates an improvement in a chromatography process useful in purifying lactoferrin and other proteins comprising the steps of (a) contacting a substance with a first adsorbent to obtain adsorbed and non-adsorbed fractions, (b) eluting the adsorbed fraction with
30 an eluant and (c) contacting the adsorbed fraction with a second adsorbent, wherein the improvement comprises equilibrating the second adsorbent with the eluant followed

by contacting the eluate containing the adsorbed fraction with the second adsorbent.

Fig. 1 is a schematic diagram showing a preferred embodiment of the improved chromatography method of the present invention. Fig. 2 is a flow chart showing a preferred embodiment of purifying lactoferrin in accordance with the present invention.

Lactoferrin is produced according to the present invention using recombinant DNA technology. That is, by using recombinant DNA technology, a polypeptide containing the primary structural conformation of naturally occurring human lactoferrin and possessing its biological properties is produced. The preferred source of DNA encoding lactoferrin is a cDNA library derived from human RNA and ligated to an appropriate vector according to methods that will be readily apparent to the skilled artisan, e.g., as disclosed in Davis, et al., Basic Methods in Molecular Biology, Elsevier Science Publishing Co., Inc, (1986), the disclosure of which is incorporated herein by reference. In a preferred embodiment, the RNA is isolated from the human mammary gland and the vector is phage λ gt 11. The cDNA library is screened for positive (DNA carrying the lactoferrin gene) clones using techniques that will be readily apparent to the skilled artisan, such as disclosed in the aforesaid Davis, et al., publication and Rado, et al., Blood, 70, No. 4 (October, 1987) pp. 989-983, the disclosure of which is incorporated herein by reference. For example, the cDNA is hybridized to a radiolabeled oligonucleotide probe and the positive clones identified by autoradiography. Preferably, positive clones are identified using lactoferrin antisera, and the antisera-containing clones are recognized using an appropriate development system, such as an avidin/biotin reaction system. Large numbers of positive clones are then generated by

infecting an appropriate bacterial host, such as E. coli Y 1090, using methods that will be readily apparent to the skilled artisan such as disclosed in the aforesaid Davis, et al., publication. DNA is then isolated from the clones. The
5 cDNA encoding lactoferrin is then cut with an appropriate restriction endonuclease such as EcoRI. The cut DNA encoding lactoferrin is separated by chromatography. Preferably, the separated cDNA is further sub-cloned into another vector, such as the pGEM-4 plasmid, and the inserted cDNA again
10 excised and separated.

Expression of human lactoferrin according to the present invention is performed using an appropriate expression vector, such as the plasmid pAO804, and an appropriate host organism, such as the yeast Pichia pastoris. Other useful
15 hosts include a mammalian cell line such as Chinese hamster ovary (CHO) and expression vectors that will be readily apparent to the skilled artisan. Insertion of the cDNA and expression of the human lactoferrin are carried out according to techniques that will be readily apparent to the skilled
20 artisan, such as disclosed in Rothstein, Methods in Enzymology, 101, 202-210 (1983), and Tschopp, et al., Bio/Technology, 5, 1305-1308 (1987), the disclosures of which are incorporated herein by reference.

Purification of the expressed protein according to the
25 present invention is preferably carried out by one of several methods. In one preferred embodiment, cell-free culture media containing the expressed lactoferrin is passed through a filter that retains material having a molecular weight greater than about 10,000 daltons and then sterilizing the
30 retained protein. The material retained by the filter is subjected to a two-step affinity chromatography process. In the first step, the affinity ligand is the reactive dye Cibacron blue F3G-A (color index (C.I.) 61211, λ max

605(374)nm) disclosed in Bezwoda, et al., Clin. Chim. Acta., 157, 89-94 (1986), and Chemical Abstracts Service (CAS) No. 12236-82-7, the disclosures of which are incorporated herein by reference. Cibacron blue F3G-A can be covalently bound to a cross-linked agarose gel by the triazine coupling method as described in Bohme, et al., J. Chromatography, 69, 209-214 (1972), the disclosure of which is incorporated herein by reference. In the second step, controlled-pore glass (CPG) or silicic acid is used to further purify the adsorbed material obtained in the first step. In another preferred embodiment, the adsorbed material from the CPG or silicic acid is further chromatographed in a third step using one of the following chromatography techniques before final filtration and sterilization: T-Gel chromatography; immobilized metal-ion affinity chromatography (IMAC) using a metal ion capable of forming a complex with lactoferrin, such as nickel, which can be coupled with an imminodiacetic acid-epoxy activated gel (IDA Me(II)) in accordance with Sulkowski, Frontiers in Bioprocessing, Sidkar et al., ed., 343-353 (1990), the disclosure of which is incorporated herein by reference; or chromatography with the ligand phenyl glycidyl ether, which can be coupled to a cross-linked agarose gel as disclosed in Janson and Riden, Protein Purification Principles High Resolution Methods and Applications, VSH Publishers New York (1989), incorporated by reference herein. The two-step and three-step methods are schematically presented by the flow chart in Fig. 2.

The improved chromatography process of the present invention is useful in purifying proteins, such as lactoferrin produced in accordance with the present invention. As shown in Fig. 1, crude fermentation broth contained in tank 1 passes to permeable membrane 3, which retains material having a molecular weight greater than

10,000 daltons and passes an ultrafiltrate containing water, salts, and low-molecular-weight proteins. The retained material is washed with a buffer and further concentrated. The washed material is then applied to chromatography column 5 containing an adsorbent that has been equilibrated with the buffer used to wash the filtered material while valve 7 is open and valve 9 is closed. After non-adsorbed material is discharged through valve 7, valve 7 is closed and valve 9 opened. Adsorbed material is then eluted, and the eluate passed directly to the second column 11, containing an adsorbent previously equilibrated with the eluant used to elute the adsorbed material. Use of the same medium to elute material from the adsorbent in column 5 and equilibrate the adsorbent column 11 avoids the need for timely and involved medium exchange procedures between the two adsorption steps. Passage of the adsorbed material through column 11 occurs while valve 13 is open and valve 15 is closed. Eluting adsorbed material from column 11 occurs while valve 13 is closed and valve 15 is open, thereby passing eluate from column 11 directly to a filter (not shown) capable of retaining material having a molecular weight of at least 10,000 daltons. Although demonstrated for use in purifying lactoferrin, the aforesaid method and apparatus is contemplated in other tandem chromatography procedures that will be readily apparent to the skilled artisan. For example, the invention is useful in purifying proteins with similar hydrophobicity to lactoferrin.

The nutritional supplement of the present invention contains an effective amount of lactoferrin loaded with one or more trace elements, either alone or in combination with one or more nutritionally acceptable carriers or diluents. Preferred nutritional supplements include tablets, gelatin capsules, or liquids containing the lactoferrin together with

diluents, such as lactose, dextrose, sucrose, mannitol, sorbitol, cellulose, and glycine; binders, such as magnesium aluminum silicate, starch paste, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, and polyvinylpyrrolidone; disintegrants, such as starches, agar, alginic acid or its sodium salt, and effervescent mixtures; as well as absorbents, colorants, flavors and sweeteners. Alternatively, the trace-element loaded lactoferrin can be added to foods such as baby formula, cereal, and ice cream to enhance the nutritional value of the food. The preferred amount of trace element-loaded lactoferrin in the supplement based on the weight of 1g of the supplement is 5-50 mg, more preferably 20-30 mg, most preferably 25 mg. An effective daily amount of trace element-loaded lactoferrin varies, based on the individual, from about 10-30 mg, preferably 20-30 mg, more preferably 25 mg. The preferred trace element in the supplement is iron, but other trace elements, e.g., zinc and copper, are contemplated within the scope of the present invention. Loading lactoferrin with the appropriate trace element is accomplished by simple titration with, e.g., ferrous ammonium in the presence of bicarbonate, according to methods that will be readily apparent to the skilled artisan. Preferred loading is such that at least 35%, more preferably at least 50%, most preferably at least 70%, of the metal-binding sites are bound to the trace element. The lactoferrin contemplated for use in the supplement is preferably of human derivation, more preferably via DNA recombinant means, but other lactoferrins, such as bovine and porcine lactoferrin, are contemplated.

Lactoferrin can be used as an antiseptic in accordance with the present invention either alone or in the form of a powder, solution, ointment, aerosol spray, or cream to any part of the subject as an aid in the prevention or treatment

of microbial infections. By depriving the surrounding environment of iron, lactoferrin inhibits the growth of microbes, such as bacteria. Preferable antiseptics of the present invention include lactoferrin either alone or
5 compounded with carriers such as, saline silica, talcum, stearic acid, its magnesium or calcium salt, polyethyleneglycol, and fatty emulsions and suspensions that will be readily apparent to the skilled artisan. The lactoferrin is preferably present in the antiseptic based on
10 1 ml of the carrier at 0.1-2 mg, preferably 0.2-2 mg. An effective amount of the lactoferrin varies depending on the individual treated, severity of infection, if any, and the area to which administration is contemplated. Preferably, in treating mammals a twice-daily administration of 0.1-
15 2 mg, more preferably 0.5-2 mg, most preferably 1 mg, of lactoferrin per 0.1 square meter effected area is contemplated. For use as an antiseptic in accordance with the present invention the lactoferrin preferably has less than 25% of its metal-binding sites loaded, i.e., at least
20 75% of its metal-binding sites are available to sequester iron. More preferably, less than 20% of the metal-binding sites are loaded, most preferably less than 10%.

Lactoferrin can be applied to food (either solid or liquid) to retard spoilage in accordance with the present invention
25 either alone or compounded with any of the aforesaid nutritionally acceptable carriers or diluents. By sequestering iron, and thereby suppressing its catalytic activity, the lactoferrin reduces the iron available for either microbial multiplication or the production of
30 potentially cell-damaging free-radicals that are formed in iron catalyzed reactions from hydrogen peroxide or superoxide. For example, the lactoferrin is particularly useful in inhibiting rancidity in meat, which is iron-

dependent lipid peroxidation. To inhibit microbial growth, particularly in liquid foods such as beer and wine, the lactoferrin can be added directly to the liquid or used to coat filters through which the liquid food passes during processing. An effective amount of the lactoferrin for retarding spoilage varies depending on the type and amount of food contemplated. Preferably, the amount of lactoferrin applied to food in accordance with the present invention varies from 0.1-1 mg/ml of food with which it is mixed, or based on the surface area of the filter or solid food to which it is applied from 0.1-1 mg/6 cm². The preferred amount of lactoferrin compounded with a carrier in a food additive for retarding spoilage varies based on 1 ml of the carrier from 0.1-2 mg, preferably 0.2-2 mg. The same amounts of metal loading preferred for the antiseptic of the present invention are preferred for the food-spoilage retardant.

The antiseptic, dietary supplement, and food-spoilage retardant of the present invention can be sterilized and/or contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating osmotic pressure and/or buffers. In addition, they may also contain other therapeutically valuable substances. Said compositions are prepared according to conventional mixing, granulating, and coating methods.

To more clearly describe the present invention, the following non-limiting examples are provided. In the examples all parts and percentages are by weight unless indicated otherwise.

EXAMPLE 1

In this example, human lactoferrin is obtained from a genetically altered organism. Using breast tissue excised during the mastectomy of a woman during the eighth month of pregnancy, a human mammary gland genomic library (cDNA

ligated to λ gt 11) is prepared according to Gubler, et al., Gen., 40, 1-8 (1983) (available from Clontech, California).

5 The library is transferred onto agar plates containing a high density of E. coli Y 1090 (available from Clontech, California) (5×10^4 plaques per 90 mm plate or 1.4×10^5 plaques per 150 mm plate. The plates are allowed to stand for 3.5 hours at 42°C to obtain a lytic growth of the phage. The plates are then overlaid with nitrocellulose filters (available from Schleier and Schnell Inc. Woburn, MA, under
10 no. BA 85 NC) and heated in an incubator at 37°C for 3.5 hours.

Positive clones (i.e., containing the cDNA) are identified on the membranes using rabbit antibody to natural human lactoferrin purified in accordance with Example 8 herein.
15 Nitrocellulose filters are removed from the plates after plaque transferral, and the coated with the antibody purified in accordance with Example 8, which hybridizes with positive plaques. Following removal of excess antibody, positive plaques are developed by first applying an anti-rabbit IgG
20 conjugated with biotin (available from Sigma Chemical Co., St. Louis, MO), and then, following removal of excess biotin conjugate, applying avidin conjugated with horse radish peroxidase (available from Sigma Chemical Co., St Louis, MO). Finally, the positive plaques are identified in the reaction
25 catalyzed by horse radish peroxidase using as an enzyme substrate 4-chloro-1-Naphtol.

The positive plaques are then used to infect E. Coli Y 1090 to produce large amounts of phage in accordance with procedures set forth in Davis, et. al., supra. The resulting
30 bacteriophage is purified using 10% polyethylene glycol and DNA is isolated from the phage according to the procedures disclosed in Kislow, N.A.R., 14, 6767 (1986), the disclosure of which is incorporated herein by reference. Following the

procedures in Davis, et al., supra, the cDNA insert encoding lactoferrin is sub-cloned as follows: the cDNA insert is cut out from the phage DNA using EcoRI and purified using a high resolution ion-exchange chromatography column (Gen-Pak™ Fax available from Millipore Corporation, Waters Chromatography Division, Milford, MA). The thus purified cDNA insert is ligated using T4 DNA ligase into plasmid pGEM-4 (available from Promega, Madison, WI, and described in Yanish-Perron et al. (1985), GEN, 33, 103-109) that has been cut using EcoRI using standard techniques. The plasmid containing the insert is then transferred into E. coli JM109 (available from Promega, Madison, WI, and described in Hanahan, J. Mol. Biol., 166, 557 (1983)). The bacteria are transferred to agar plates containing ampicillin and the positive colonies grown. The plasmid is then isolated and the cDNA insert is cut from the plasmid using EcoRI and purified by ion-exchange chromatography as described above.

The cDNA insert is then ligated into the Pichia pastoris expression vector pAO804 (using the P. pastoris GTS 115 strain) so as to be flanked by the 5' and 3' regulatory sequences of the methanol-induced alcohol oxidase gene (AOX1) of P. pastoris in accordance with the procedures described in Sreekrishna, et al., Biochemistry, 28, 4117-4125 (1989), and Rothstein, Methods in Enzymology, 101, 202-210 (1983), the disclosures of which are incorporated herein by reference. The thus modified vector is then grown in minimal media as described in Creeg, et al., Mol. Cell. Biol., 5, 3376-3385 (1985), the disclosure of which is incorporated herein by reference. Following the procedure of Hagenson, et al., Enzyme Microb. Technol., 11, 650-656 (1989), the cells are grown to an OD₆₀₀ of about 1.0 and then harvested, and washed with and suspended in minimal methanol media at an OD₆₀₀ of about 4.0. The culture is held at 30°C while

12

maintained at a pH of 5.0 by adding NH_3 gas to the air stream. Expressed lactoferrin is recovered from the supernatant of the fermentation media following 15' centrifugation at 5000 rpm using a Beckman J-21B with a Rotor JA 14.

EXAMPLE 2

In this example, human lactoferrin is purified in accordance with the present invention. One liter of the supernatant from Example 1 is adjusted to about 4°C and filtered under pressure through a polysulfone ultrafiltration membrane having a pH operating range of 1-14 on a polypropylene mesh support (Pellicon™ Cassette filter System assembled with Procon pump and PTGC membrane available from Millipore) to retain proteins in excess of about 10,000 molecular weight. Pressure with simultaneous circulation is applied until 900 ml of ultrafiltrate is collected. A flow rate of about 100 ml per minute is maintained during the filtration process. The retained material (100 ml) is diluted with 900 ml 20 mM phosphate buffer (pH 7.4) and re-filtered, which is repeated four times (final exchange ratio = 10,000). The final material retained is sterilized (0.22 μm Gelman™ filter).

EXAMPLE 3

In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using affinity chromatography in which the affinity ligand is the reactive dye Cibacron blue F3G-A. The sterilized material obtained in Example 2 is adjusted to a pH of 7.5 and a final concentration of sodium chloride of 0.5 M. This material is then applied onto a column (5 cm x 35 cm) packed with cross-linked agarose coupled to the dye (available from Pharmacia Fine Chemicals, Upsala, Sweden, under the name Blue Sepharose™ CL-6B) and previously equilibrated with 50 mM

N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer (pH 7.5) containing 0.5 M sodium chloride. Adsorption is performed at a flow rate of 1 ml/min followed by washing the column with 2 bed volumes of the same HEPES
5 buffer. The non-adsorbed fraction is discarded and the adsorbed fraction containing lactoferrin is eluted from the column bed using 2 bed volumes of 50 mM HEPES buffer (pH 7.5) containing 1 M sodium chloride.

EXAMPLE 4

10 In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using control pore glass (CPG) chromatography. The eluate from Example 3 is applied onto a column (1.2 cm x 10 cm) packed
15 with CPG beads (CPG 00350 available from Electro-Nucleonics, Fairfield, N.J.) and previously equilibrated with 50 mM HEPES buffer (pH 7.5) containing 1 M sodium chloride. Adsorption is performed at a flow rate of 1 ml/min followed by washing the column with 2 bed volumes of the same buffer. The non-
20 adsorbed fraction is discarded, and the adsorbed fraction is eluted with 2 bed volumes of 0.25 M tetramethylammonium chloride (TMAC; pH 7.5). The eluate is filtered on a membrane capable of excluding material having a molecular weight greater than 10,000 daltons (Amicon™ YM 10). The
25 filtered material is then sterilized (0.22 μm Gelman™ filter) and frozen at -20°C.

EXAMPLE 5

In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using
30 immobilized metal ion affinity chromatography (IMAC). An imminodiacetic acid-epoxy activated gel (available from Pharmacia Fine Chemicals, Upsala, Sweden, under the name Chelating Sepharose™ 6B) is washed with water and equilibrated with 0.1 M sodium acetate buffer (pH 4.0)

containing 1 M sodium chloride. The gel is then packed into a chromatographic column (1.2 cm x 10 cm) and saturated with 4 bed volumes of the same sodium acetate buffer further containing 5 mg/ml of nickel chloride. Excess metal is washed from the column with the sodium acetate buffer, and the gel is equilibrated with 20 mM HEPES buffer (pH 7.0) containing 1 M sodium chloride and 2 mM imidazol.

The product of Example 4 is mixed with HEPES, sodium chloride, and imidazol to obtain a pH of 7.0, 20 mM HEPES, 1 M sodium chloride, and 2 mM imidazol. The mixture is applied onto the column at a flow rate of about 1 ml/min followed by washing the gel with 2 bed volumes of 20 mM HEPES buffer (pH 7.0) containing 1 M sodium chloride and 2 mM imidazol. The non-adsorbed fraction is discarded, and the adsorbed fraction containing lactoferrin is eluted using 2 bed volumes of 20 mM HEPES buffer (pH 7.0) containing 1 M sodium chloride and 20 mM imidazol.

EXAMPLE 6

In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using T-Gel affinity chromatography. T-gel adsorbent is prepared according to Porath, et al., Methods in Enzymology, 44, 19-45 (1976), the disclosure of which is incorporated herein by reference, and packed into a column (1.2 cm x 10 cm). The final product of Example 4 is adjusted to a pH of 7.5 and a final concentration as follows: 50 mM PIPES buffer (piperazine-N,N'-bis[2-ethanesulfonic acid] and 1,4-piperazinediethanesulfonic acid] buffer and 0.7 M ammonium sulfate. The adjusted material is applied on the column that has been previously equilibrated to 50 mM PIPES buffer (pH 7.5) containing 0.7 M ammonium sulfate with a flow rate of about 1ml/min. The non-adsorbed fraction containing lactoferrin is adjusted to a concentration of 0.1 M ammonium

15

sulfate and then applied to an identical T-gel column previously equilibrated to 50 mM PIPES buffer (pH 7.5) containing 1.0 M ammonium sulfate. The column is then washed with 7-8 bed volumes of 50 mM PIPES buffer (pH 7.5) containing 1.0 M ammonium sulfate, with lactoferrin being present in the non-adsorbed fraction.

EXAMPLE 7

In this example human lactoferrin is purified in accordance with a preferred embodiment of the present invention using a hydrophobic interaction chromatography on a cross-linked agarose gel coupled to phenyl glycidyl ether (Phenyl SepharoseTM CL-4B available from Pharmacia Fine Chemicals, Upsala, Sweden). The gel is packed into a column and equilibrated to 50 mM PIPES buffer (pH 7.0) containing 1 M ammonium sulfate. The product of Example 4 is adjusted to the equilibrating buffer and applied onto the column at a flow rate of 1 ml/min. The non-adsorbed fraction is discarded and the adsorbed fraction containing lactoferrin is eluted using 2 bed volumes of 50 mM PIPES buffer (pH 7.0).

EXAMPLE 8

In this example anti-lactoferrin serum is purified by affinity chromatography for use in accordance with a preferred embodiment of the present invention as described in Example 1 herein. The adsorbent substrate for affinity chromatography is prepared by cyanogen bromide activation as described by Axen et al., Nature, 214, 1302-1304 (1967). The substrate (available from Pharmacia Fine Chemicals, Upsala, Sweden, under the name CNBr-SepharoseTM-4B) is coupled to human lactoferrin, which acts as the affinity ligand, as follows. One gram of substrate is swelled with 1 mM HCl and washed with the same solvent on a sintered glass filter. Ten mg of natural human lactoferrin (available from Sigma Chemical Co., St. Louis, MO) is dissolved in 0.1 M NaHCO₃

buffer (pH 8.3) containing 0.5 M sodium chloride (coupling buffer). The resulting solution is mixed with the washed substrate gel for 2 hours, and then mixed with 0.2 M glycine buffer (pH 4.0) for 2 hours. The gel is then washed with
5 coupling buffer, followed by 0.1 M acetate buffer (pH 4.0) containing 0.5 M sodium chloride, followed again by coupling buffer to form the adsorbent. The adsorbent is packed into a column and washed with 20 mM phosphate buffer (pH 7.4) containing 0.5 M sodium chloride. Anti-lactoferrin serum
10 obtained from an inoculated rabbit (available from Sigma Chemical Co., St Louis, MO) is passed through the column at a flow rate of 1 ml/min and the non-adsorbed material discarded. Adsorbed material containing the purified protein is eluted with 2 bed volumes of 0.2 M glycine buffer (pH 2.0)
15 containing 0.5 M of sodium chloride. The eluate is neutralized with 0.1N NaOH to obtain pH 7.5 and then sterilized (0.22 μ m GelmanTM filter) and frozen at -20°C.

Claimed is:

1. Human lactoferrin expressed by recombinant DNA.
2. Lactoferrin having less than 25% metal loading.
3. The lactoferrin of claim 2 that is human lactoferrin.
- 5 4. The lactoferrin of claim 2 having less than 10% metal loading.
5. Lactoferrin having at least 35% metal loading.
6. The lactoferrin of claim 5 having at least 50% metal loading.
- 10 7. The lactoferrin of claim 5 that is human lactoferrin.
8. A genetically altered organism capable of producing human lactoferrin.
9. The organism of claim 8 that is eukaryotic.
10. The organism of claim 8 that is Pichia pastoris.
- 15 11. A process for producing human lactoferrin comprising the steps of:
 - (a) isolating DNA encoding human lactoferrin from a cDNA library;
 - (b) inserting the isolated DNA into the DNA of a host
 - 20 organism;
 - (c) culturing the host organism to express human lactoferrin; and
 - (d) recovering the expressed human lactoferrin from culture media.
- 25 12. The process of claim 11 wherein the lactoferrin is recovered by the steps of (i) filtering the culture media to retain a first material having a molecular weight of at least 10,000 daltons, (ii) chromatographing the retained material on an adsorbent having chromophore C.I. 61211 as an affinity
- 30 ligand to obtain adsorbed and non-adsorbed fractions, (iii) chromatographing the adsorbed fraction on a medium wherein the medium is controlled pore glass beads or silicic acid to obtain adsorbed and non-adsorbed fractions, and (iii)

filtering the adsorbed fraction from the medium to retain a second material having a molecular weight of at least 10,000 daltons.

5 13. The process of claim 12 further comprising the steps of (iv) chromatographing the second retained material on T-Gel at least once to obtain adsorbed and non-adsorbed fractions, and (v) filtering the non-adsorbed fraction from the T-Gel to retain a third material having a molecular weight of at least 10,000.

10 14. The process of claim 12 further comprising the steps of (iv) chromatographing the second retained material on a third adsorbent having phenyl glycidyl ether as an affinity ligand to obtain adsorbed and non-adsorbed fractions, and (v) filtering the adsorbed fraction from the third adsorbent to
15 retain a third material having a molecular weight of at least 10,000 daltons.

15 15. The process of claim 12 further comprising the steps of (iv) chromatographing the second retained material on a fourth adsorbent having an immobilized metal ion as an
20 affinity ligand to obtain adsorbed and non-adsorbed fractions, and (v) filtering the adsorbed fraction to retain a fourth material having a molecular weight of at least 10,000 daltons.

25 16. A process for inhibiting microbial growth on a mammal comprising topically applying a therapeutically effective amount of lactoferrin having less than 25% metal loading.

17. The process of claim 16 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

30 18. A process for inhibiting trace-element deficiency in a mammal comprising orally administering a nutritionally effective amount of lactoferrin having at least 35% trace-element loading.

19. The process of claim 18 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

20. The process of claim 18 wherein the trace element is at least one of iron, copper, or zinc.

5 21. A method for retarding food-spoilage comprising applying to food an effective amount of lactoferrin having less than 25% metal loading.

22. The method of claim 21 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

10 23. A nutritional supplement comprising an effective amount of lactoferrin having at least 35% trace-element loading and a nutritionally acceptable carrier or diluent.

24. The supplement of claim 23 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

15 25. The supplement of claim 23 wherein the trace element is at least one of iron, copper, or zinc.

26. A disinfectant comprising an effective amount of lactoferrin having less than 25% metal loading and an acceptable carrier or diluent.

20 27. The disinfectant of claim 26 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

28. A food-spoilage retardant comprising an effective amount of lactoferrin having less than 25% metal loading and an acceptable carrier or diluent.

25 29. The additive of claim 28 wherein the lactoferrin is human lactoferrin expressed by recombinant DNA.

30 30. In a chromatography process comprising the steps of (a) contacting a substance with a first adsorbent to obtain adsorbed and non-adsorbed fractions, (b) eluting the adsorbed fraction with an eluant, and (c) contacting the adsorbed fraction with a second adsorbent, the improvement comprising equilibrating the second adsorbent with the eluant followed

by contacting the eluate containing the adsorbed fraction with the second adsorbent.

31. The process of claim 30 wherein the first adsorbent is contained in a first column connected to a second column
5 containing the second adsorbent by a means through which the eluate passes from the first column to the second column.

32. The process of claim 31 wherein the means sequentially (i) discharges material not adsorbed on the first adsorbent from the first column and (ii) passes the eluate from the
10 first column to the second column.

33. The process of claim 32 further comprising the sequential steps of (d) discharging non-adsorbed material from the second-adsorbent, (e) eluting adsorbed material from the second adsorbent, and (f) filtering the eluate, wherein
15 the non-adsorbed material and the eluate pass through a means capable of directing the effluent from the second column to the filtering means or the discharge locus.

34. The process of claim 31 further comprising the steps of obtaining the substance by passing a mixture through a
20 filter that retains the substance, and prior to contacting the substance with the first adsorbent, washing the substance on the filter with a buffer and equilibrating the first adsorbent with the buffer.

35. A chromatography apparatus comprising (a) a first
25 column containing a first adsorbent, (b) a second column containing a second adsorbent, and (c) a means capable of (i) carrying effluent from the first column to the second column or (ii) discharging the effluent.

36. The apparatus of claim 35 further comprising (d) means
30 capable of directing effluent from the second column to at least two alternative loci.

37. In a chromatography process comprising the steps of
(a) equilibrating an adsorbent with a buffer and (b) applying
a protein-containing material to the adsorbent to obtain
adsorbed and non-adsorbed fractions, the improvement
5 comprising passing the material through a filter capable of
excluding material having a molecular weight greater than
10,000 daltons and washing the material on the filter with
the buffer before applying the material to the adsorbent.

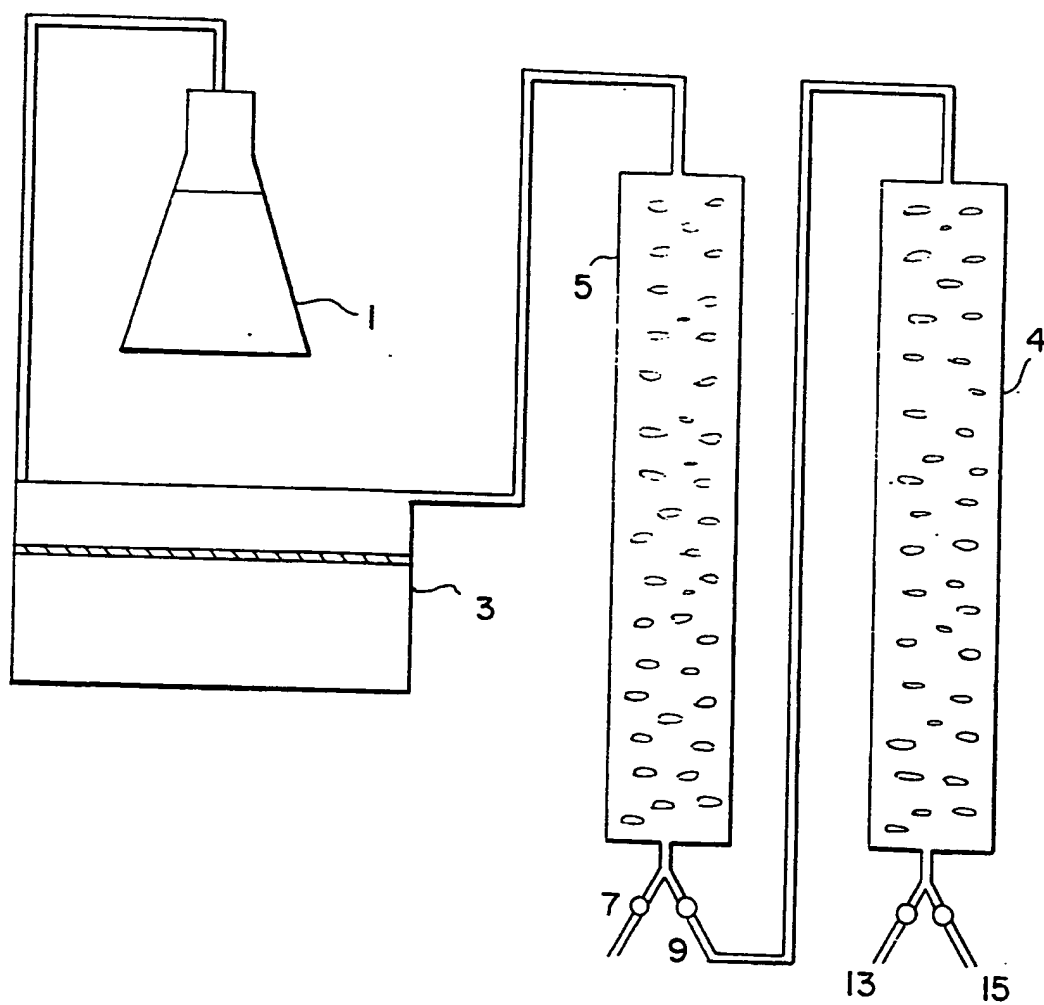
38. A process for producing human lactoferrin comprising
10 the steps of:

(a) culturing a host cell genetically altered to carry DNA
encoding human lactoferrin to express human lactoferrin and
(b) recovering the expressed lactoferrin from culture
media.

15 39. Genetically modified DNA capable of expressing human
lactoferrin in a host organism.

1 / 2

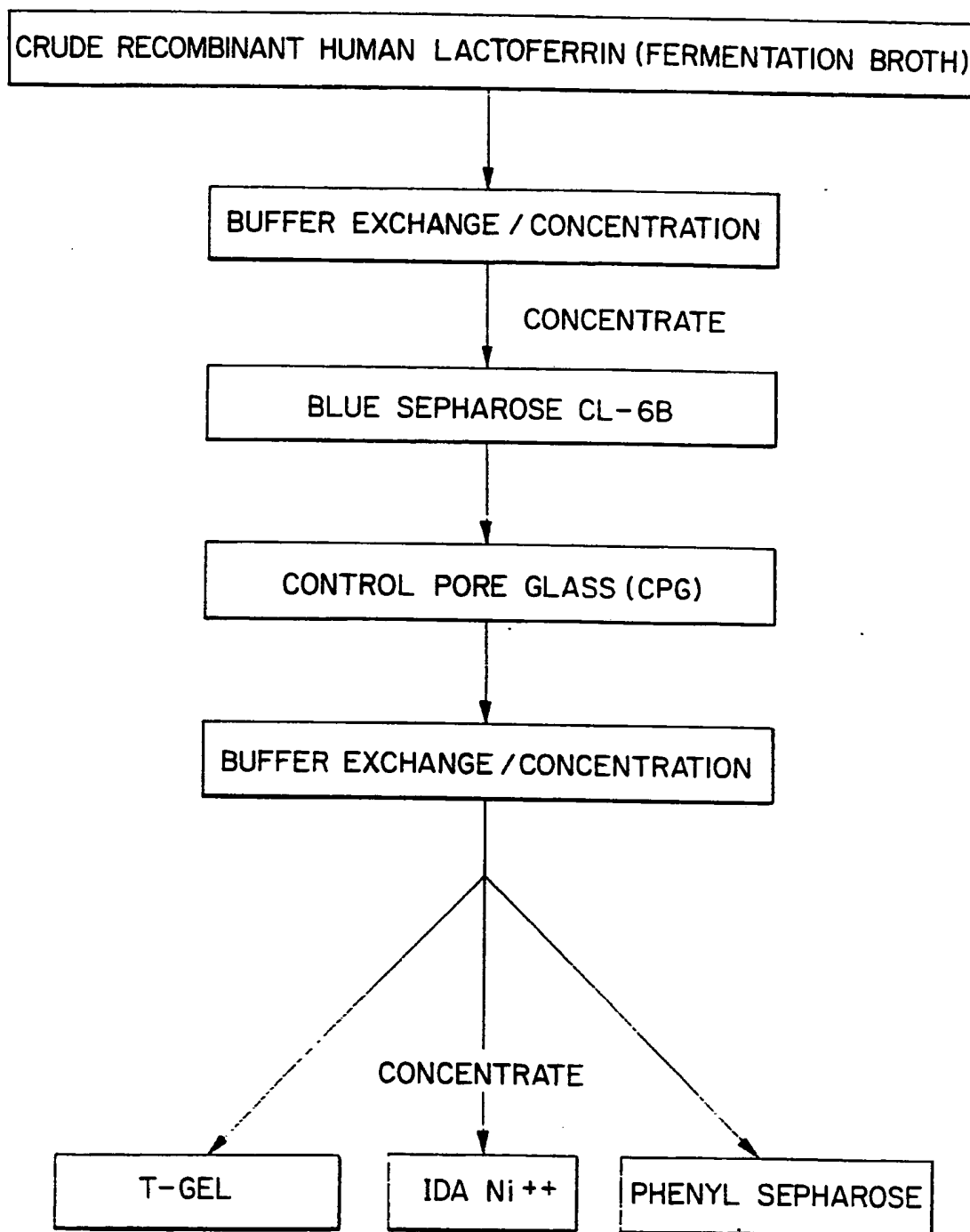
FIG. 1



SUBSTITUTE SHEET

2 / 2

FIG. 2



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01335

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC(5): C12N 15/12, 15/63; A23L 3/3463; A01N 37/18
 U.S.CL.: 435/69.1, 240.1, 252.3; 536/27; 99/485; 514/2

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
US	435/69.1, 240.1, 252.3; 536/27; 99/485; 514/2

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

CA: KEY WORDS: LACTOFERRIN, DISINFECTANT, NUTRITIONAL SUPPLEMENT, FOOD SPOILAGE RETARDANT

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	US, A. 4,436,658 (PEYROUSET ET AL) 13 March 1984. see entire document.	1-7.16-29
Y	BLOOD, Vol. 70, No. 4 issued October 1987, 1987, Rado et al. "Isolation of Lactoferrin cDNA From a Human Myeloid Library and Expression of mRNA During Normal and Leukemic Myelopoiesis", pages 989-993, see entire document.	8-15, 38-39 17, 19, 22, 24, 27, 29
Y	BIOCHEMISTRY Vol. 28, NO. 9, issued 1989, SREEKRISHNA ET AL. "High-Level Expression, Purification, and Characterization of Recombinant Human Tumor Necrosis Factor Synthesized in the Methylophilic Yeast <i>Pichia pastoris</i> ", pp 4117-4125, see entire document.	10

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Δ" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

21 MAY 1991

International Searching Authority

ISA/US

Date of Mailing of this International Search Report,

26 JUL 1991

Signature of Authorizing Officer

Sharon L. Nolan

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	<u>TIBTECH</u> , Vol. 5 issued August 1987, Porath et al "Thiophilic interaction and the selective adsorption of proteins", pp. 225-229, see entire document.	12-15
Y	Sulkowski, "Protein Purification: Micro to Macro" published 1987 (Department of Molecular and Cellular Biology, Roswell Park Memorial Institute, Buffalo, N.Y), pp. 177-195, see entire document.	12-15
Y	<u>Clinica Chimica Acta</u> , Vol. 157, issued 1986, Bezwoda et al, "Isolation and Characterisation of lactoferrin separated from human whey by adsorption chromatography using Cibacron Blue F3G-A linked affinity adsorbent", pages 89-93, see entire document.	12-15 30-37

FURTHER INFORMATION

CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE :

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING :

This International Searching Authority found multiple inventions in this international application as follows:

- I) Product (cl. 1-7), Method of making recombinantly (cl. 8-15 & 38-39), + Method of use for inhibiting microbial growth (cl. 16-17 & 25-27).
- II) Method of making by purification (cl. 30-37) III) Method of use as nutritional supplement (cl. 18-20, 23-24) IV) Method of use as food spoilage retardant (cl. 21-22, 28-29).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.